Volume 277, number 1,2, 253–256 FEBS 09274 December 1990

Reduction of HSV-1 binding to BHK cells after treatment with phosphatidylinositol-specific phospholipase C

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Received 31 August 1990; revised version received 2 November 1990

Baby-hamster kidney cells were treated with unspecific and phosphatidylinositol-specific phospholipase C (PI-PLC) prior to infection with herpes simplex virus type 1 or 2. Subsequent to PI-PLC treatment, a 30% reduction of infectivity and receptor binding was observed for type 1 virus, while type 2 was unaffected. Treating the cells with unspecific phospholipase C did not affect subsequent infection with either virus. Treatment of the virus particles with the unspecific phospholipase reduced its infectivity, probably due to loss of the viral envelope. PI-PLC treatment of virus particles did not have any such effect on virus infectivity.

Herpes simplex virus type 1; Receptor; Phosphatidylinositol-anchoring; Phosphatidylinositol-specific phospholipase C

1. INTRODUCTION

Anchoring of cell surface proteins to the plasma membrane through covalent binding to inositol-containing phospholipids in the membrane has been demonstrated for a number of proteins (reviewed in [1]). Among these are heparan sulphate proteoglycans, which in their plasma membrane form can be phosphatidylinositol (PI)-anchored [2,3]. We have previously demonstrated that polycations such as neomycin and polylysine inhibit binding of herpes simplex virus type I (HSV-1) to its cellular receptor by interference with receptor function [4,5]. Since neomycin is known to bind strongly to phosphoinositides, as shown by nuclear magnetic resonance studies [6], and polylysine interferes with the known PI-anchored membrane protein acetylcholinesterase [7], PI-anchoring of the HSV-1 receptor was a possible explanation of our results. Moreover, the finding that the HSV-1 receptor is most probably a heparan sulphate-containing cell surface protein [8] made this possibility even more likely. In this paper we demonstrate that the HSV-1 receptor is at least partially anchored to the plasma membrane via a PI-anchor in baby-hamster kidney cells.

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Abbreviations: PI, phosphatidylinositol; PC, phosphatidylcholine; PLC, phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; BHK cells, baby-hamster kidney cells

2. MATERIALS AND METHODS

2.1. *Materials*

Eagle's minimum essential medium (EMEM) and newborn calf serum were purchased from Flow Laboratories (Irvine, Ayrshire, UK). Cell culture dishes were purchased from Nunc (Roskilde, Denmark). [^{32}P]P_i (10 mCi/ml, carrier-free) and [^{3}H]inositol (1 mCi/ml) were from Amersham International (Amersham, Bucks., UK). Unspecific phospholipase C (PLC) was from Sigma Chemical Co. (St. Louis, MO, USA) and the PI-specific PLC (PI-PLC) from Funakoshi Pharmaceutical Co. (Tokyo, Japan). For preliminary experiments, the PI-PLC used were the generous gifts from Dr H. Ikezawa (Nagoya City University, Japan) and Dr Martin Low (Columbia University, New York, USA). Thin layer chromatography plates and chemicals were obtained from Merck (Darmstadt, Germany).

2.2. *Cells and virus*

Baby-hamster kidney (BHK) 21 clone 13 cells were grown until confluence in 32 mm plastic dishes in EMEM supplemented with 10% newborn calf serum. The virus strains used were HSV-1 strain 17syn+ and HSV-2 strain HG52. [^{35}S]Methionine-labelled virus was prepared as described elsewhere [5]. ^{32}P-Labelling of virions was performed similarly, with 50 μCi label present per ml medium as the virus was propagated in BHK cells. Virions were harvested and purified as described [9]. [^{3}H]Inositol-labelling of BHK cell was performed by growing the cells for 48 h in the presence of medium supplemented with 10 μCi/ml [^{3}H]inositol. Labelled medium was removed and cells were washed extensively with phosphate-buffered saline before PLC-treatment. ^{32}P-Labelling was performed similarly, except that 5 μCi [^{32}P]P_i was present for 24 h, and removed from the medium before experiments were started. Before PLC-treatment, ^{32}P-labelled cells were disrupted by sonication in order to make a major part of the labelled phospholipids available to the enzyme. In plaque assays and virus binding experiments, cells were intact so that infection could be assayed.

2.3. *PLC treatment of cells and viruses*

Confluent BHK cells were treated with various concentrations of PLC for 1 h at 37°C, before the enzyme-containing medium was carefully removed. In virus titration assays, approximately 50 plaque-

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00145793/90/$3.50

forming units of virus was added per dish. After a 1 h adsorption period, medium was changed and incubations continued for 48 h, at which time plaque formation was recorded. When [^{32}S]methionine-labelled virus was added, adsorption was assayed at 4°C. After 1 h incubation, cells were washed extensively with phosphate-buffered saline, and bound radioactivity was measured. Unspecific binding was subtracted and defined as the binding of radiolabel obtained in the presence of 2000 times excess of cold virus. Experiments were performed so that specifically bound radioactivity was in the range of 5000–10 000 cpm per dish.

When the virus was PLC-treated, stock solutions of HSV-1 or HSV-2 were treated with various concentrations of PLC for 1 h at 37°C. The virus solution was then diluted by 10^5 in medium before it was added to the cells in plaque assays. Cells were thus exposed to PLC-concentrations (for the 1 h adsorption period) far below the ones which were effective on cells. All experiments were performed with triplicate incubations at each point. Each experiment was repeated 4 times, yielding similar results.

2.4. *Phospholipid extraction and separation*

A modification of the extraction procedure described by Bligh and Dyer [10] was used, as previously described [11]. Individual phospholipids were separated in a chloroform/methanol/40% methylamine/water (60:36:5:5 v/v) solvent system as described by de Chaffoy de Courcelles et al. [12]. Radiolabelled phospholipids were detected by autoradiography.

3. RESULTS

3.1. *Treatment by unspecific phospholipase C*

First, the effect of treating either host cells or virus with unspecific PLC prior to infection was assayed. Generally, the acidic phospholipids such as phosphatidyl serine, phosphatidylinositol and its phosphorylated derivatives were unaffected by the unspecific PLCs tested, while the remaining major phospholipids were degraded by these enzymes (data not shown). Fig. 1A and C shows the enzymatic degradation of phosphatidyl choline (PC) and phosphatidylinositol (PI) in sonicated host cells (A) and virions (C). Both in the cells and the virions, the unspecific PLC degraded PC at moderate PLC concentrations, while PI was unaffected.

Next, the effect of PLC treatment on HSV-1 and HSV-2 infection was assayed. As shown in Fig. 1B, unspecific PLC treatment of intact cells, had no significant effect on subsequent infection by either HSV-1 or HSV-2 in the range of PLC concentrations tested. In-

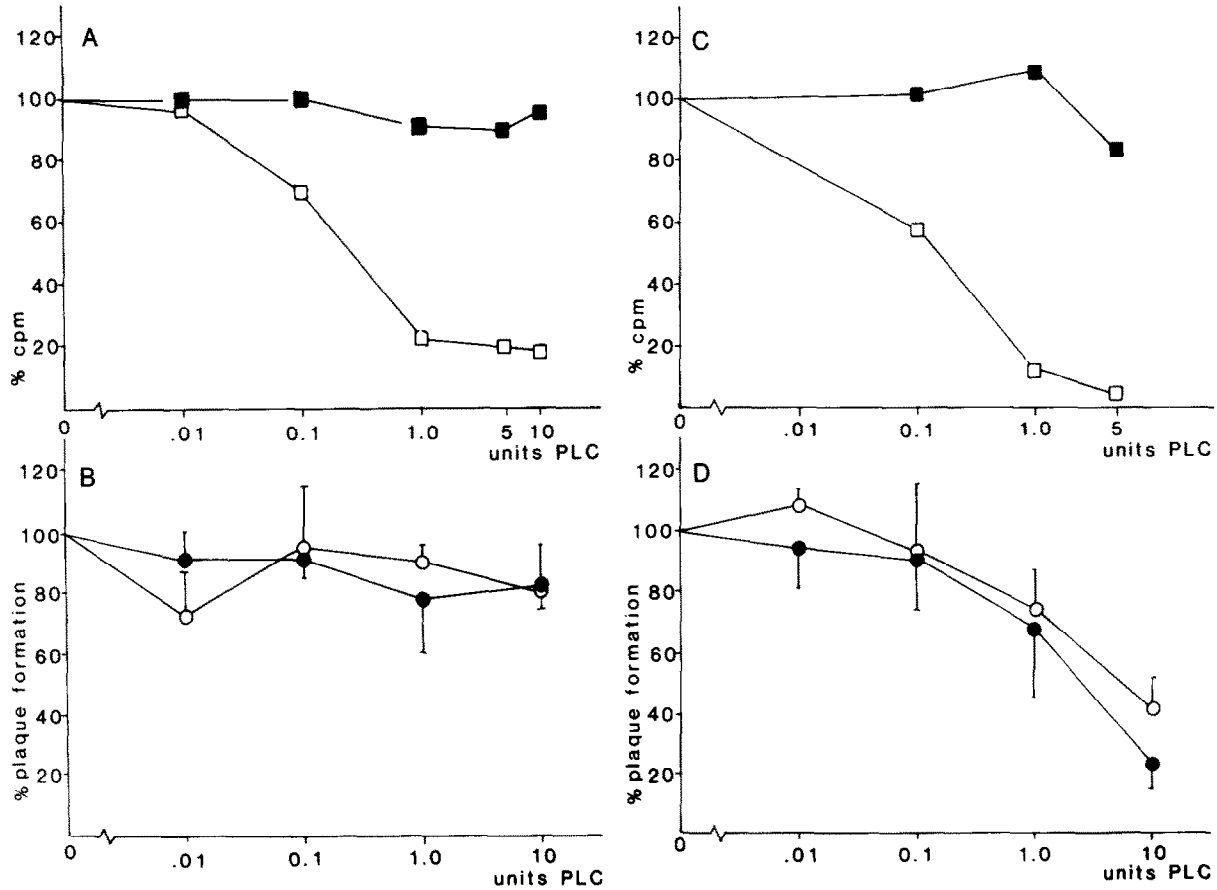

Fig. 1. Effect of unspecific PLC treatment on phospholipid hydrolysis and HSV infectivity. Host cells (A and B) or virions (C and D) were treated with the indicated amounts of PLC for 1 h at 37°C before subsequent extraction of ^{32}P-labelled phospholipids (A and C) or HSV-1/HSV-2 infection of the cells (B and D). The hydrolysis of PC (open squares) and PI (closed squares) are indicated (A and C) as percentage of cpm obtained in the absence of enzyme. Panels B and D show plaque numbers after infection with HSV-1 (closed circles) or HSV-2 (open circles) as percentage of the plaque numbers obtained in the absence of enzyme. Data are means of triplicate determinations.

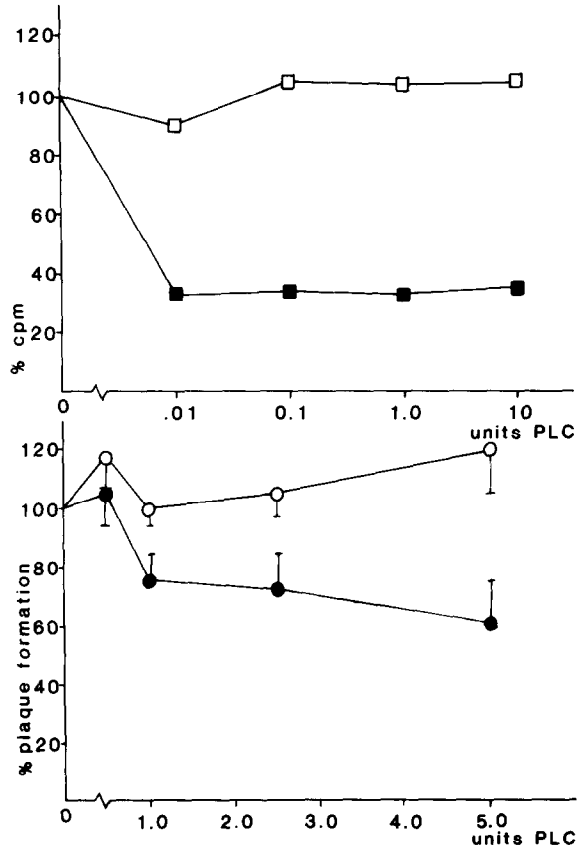

Fig. 2. Effect of PI-PLC treatment of host cells on phospholipid hydrolysis and HSV infectivity. The upper panel shows hydrolysis of [^{32}P]PC (open squares) and [^{32}P]PI (closed squares). The lower panel shows the plaque numbers after infection with HSV-1 (closed circles) or HSV-2 (open circles). Data presentation and experimental conditions were as described in the legend to Fig. 1.

fection was assayed as plaque numbers, i.e. complete infection cycles. Unspecific PLC-treatment of HSV-1 and HSV-2 (Fig. 1D) did, on the other hand, reduce infectivity at concentrations corresponding to those which degraded PC.

3.2. *Treatment by PI-specific PLC*

PI-PLC treatment of virus particles did not affect subsequent infection of BHK cells at doses up to 5 units PLC per ml (data not shown). PI-PLC did selectively degrade PI both of virions (not shown) and cells (Fig. 2, upper panel). The data presented in Fig. 2 (lower panel) also show that PI-PLC treatment of cells inhibited subsequent infection of HSV-1, while HSV-2 infection was unaffected. The concentrations necessary for full effect were, however, higher than those yielding maximal degradation of PI (Fig. 2, upper and lower panels).

PI-PLC treatment of BHK cells was also performed in experiments where subsequent binding of radiolabelled HSV-1 or HSV-2 was assayed (Fig. 3). While 2.5 U PI-PLC inhibited binding of [^{35}S]HSV-1 by approximately 30% [^{35}S]HSV-2 binding was not significantly affected.

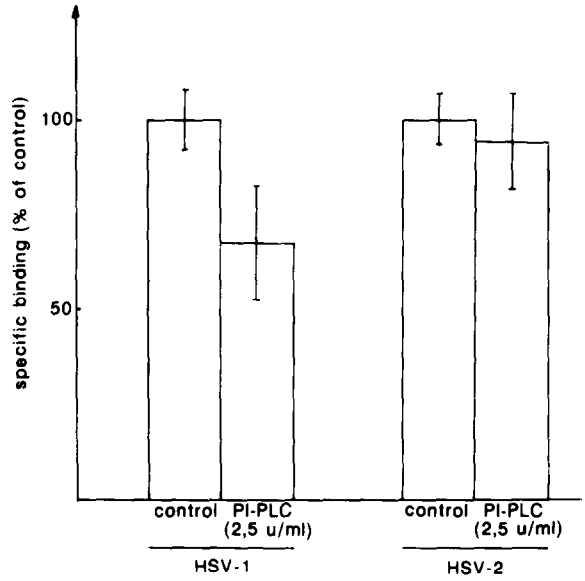

Fig. 3. Effect of PI-PLC treatment of BHK cells on subsequent binding of ^{35}S-labelled HSV-1 and HSV-2. Binding was assayed at 4°C. Control binding was the specific radioactivity obtained in the absence of enzyme. Data are means ±SD for triplicate determinations.

4. DISCUSSION

PI-PLC treatment of intact cell is considered to specifically release PI-containing compounds, and release of proteins after enzyme treatment thus being evidence for PI-anchoring [1]. A number of proteins have been shown to be attached to the membrane in this way. Among these are heparan sulphate-containing proteoglycans, which in their plasma membrane form have been shown to be at least partially PI-linked; in ovarian granulosa cells of rats, approximately 25% of the plasma membrane heparan sulphate proteoglycans were found to be PI-anchored [13]. Based on the report that heparan sulphate is an essential part of the HSV receptor [8], this raised the possibility of PI-anchoring of the HSV receptor. The observations that PI-PLC treatment of BHK cells reduced subsequent HSV-1 infection by approximately 30% in plaque assays (Fig. 2) as well as in binding assays (Fig. 3), both support this. The specificity of the observed enzymatic degradation is confirmed both by testing degradation of individual phospholipids (Fig. 1A and C, Fig. 2), and by the lack of effect of unspecific PLC on infection (Fig. 1B). The finding that the unspecific PLC affected the infectivity of both HSV-1 and HSV-2 (Fig. 1D) was not unexpected, as enveloped viruses all depend on their intact membranes for infectivity.

The finding that PI-PLC treatment only partially reduced binding and infection of HSV-1 could be explained in various ways. Since the observed 30% reduction in binding of HSV-1 coincides remarkably well with the 25–30% PI-anchoring of plasma membrane

heparan sulphate proteoglycans observed in other cells, our observations could be caused by binding of the virus to all heparan sulphate present on the cell surface, whether it was PI-linked or not, while only the PI-linked fraction was degraded.

The limited effect of the PI-PLC in our assays could just as well be caused by inefficiency of the PI-PLC in degrading the anchors, as has been shown in other systems, such as for the Thy-1 antigen [14] and the human erythrocyte acetylcholinesterase [15]. Both of these are PI-anchored proteins, but the anchors are in some way less available to the enzyme.

The recent report that HSV-1 binding to the fibroblast growth factor receptor could account for 70% of HSV-1 binding in certain cells [16], could be an alternative explanation of our finding of only 30% reduction of binding after PI-PLC treatment. Alternatively, the PI-anchoring may not be of the receptor molecule, but of a protein with some 'supportive' function in the adsorption process.

The finding that HSV-2 receptor binding was unaffected by PI-PLC treatment was interesting. Previous results indicated that in our cell system, HSV-1 and HSV-2 bind to separate receptors [17]. The results presented in this paper support these findings.

Acknowledgements: The financial support of the Norwegian Research Council for Science and the Humanities, and the Norwegian Cancer Society is acknowledged. We wish to thank Dr Lars Haarr for critical reading of the manuscript.

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